Carbonic Anhydrase Is Essential for \textit{Streptococcus pneumoniae} Growth in Environmental Ambient Air

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Carbonic Anhydrase Is Essential for *Streptococcus pneumoniae* Growth in Environmental Ambient Air

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The respiratory tract pathogen *Streptococcus pneumoniae* needs to adapt to the different levels of carbon dioxide (CO2) it encounters during transmission, colonization, and infection. Since CO2 is important for various cellular processes, factors that allow optimal CO2 sequestering are likely to be important for pneumococcal growth and survival. In this study, we showed that the putative pneumococcal carbonic anhydrase (PCA) is essential for in vitro growth of *S. pneumoniae* under the CO2-poor conditions found in environmental ambient air.

Enzymatic analysis showed that PCA catalyzes the reversible hydration of CO2 to bicarbonate (HCO3⁻), an essential step to prevent the cellular release of CO2. The addition of unsaturated fatty acids (UFAs) reversed the CO2-dependent in vitro growth inhibition of *S. pneumoniae* strains lacking the pca gene (Δpca), indicating that PCA-mediated CO2 fixation is at least associated with HCO3⁻ dependent de novo biosynthesis of UFAs. Besides being necessary for growth in environmental ambient conditions, PCA-mediated CO2 fixation pathways appear to be required for intracellular survival in host cells. This effect was especially pronounced during invasion of human brain microvascular endothelial cells (HBMEC) and uptake by murine J774 macrophage cells but not during interaction of *S. pneumoniae* with Detroit 562 pharyngeal epithelial cells. Finally, the highly conserved pca gene was found to be invariably present in both CO2-independent and naturally circulating CO2-dependent strains, suggesting a conserved essential role for PCA and PCA-mediated CO2 fixation pathways for pneumococcal growth and survival.

The Gram-positive bacterium *Streptococcus pneumoniae*, or pneumococcus, is a human respiratory tract pathogen that contributes significantly to global mortality and morbidity. In addition, it is an important asymptomatic colonizer of the human nasopharynx, with carriage rates around 10% in adults and over 40% in children (6). Pneumococcal colonization and infection are closely linked, but knowledge of the factors that contribute to transmission, carriage, disease, and transition from carriage to disease is still limited. Research on components that physically contribute to host-pathogen interactions, such as capsular polysaccharides, adhesins, and toxins, has provided valuable insights into the process of pneumococcal pathogenesis (20). In contrast, the influence of environmental factors on pneumococcal growth and survival remains fairly unexplored.

*S. pneumoniae* needs to adapt to various aerobic and anaerobic conditions, reflecting the different niches it occupies during transmission, colonization, and invasive disease. During niche transition, oxygen (O2) levels change considerably. Levels of O2 are 21% in ambient air, decrease to 10 to 15% in the alveoli of the lungs, and are about 5% in resting cells. In O2-rich conditions, *S. pneumoniae* expresses pyruvate oxidase (SpxB), which generates acetyl-phosphate as a source of ATP and hydrogen peroxide (H2O2) for interspecies competition at the mucosal surfaces of the nasopharynx (41). The presence of O2 is also a prerequisite for the pneumococcal X state (4, 14), which is a physiological condition that allows for genetic transformation and an adequate response to environmental stress (32). Recently, it was shown that the fatty acid (FA) content of the pneumococcal cell membrane (31) and the expression of 69 genes (8) change in response to the availability of O2. Finally, changes in O2 levels can also affect production of the polysaccharide capsule (48), which is the major pneumococcal virulence determinant.

Similar to those of O2, the levels of carbon dioxide (CO2) vary considerably among the different pneumococcal niches inside and outside the host. Ambient levels of CO2 in the environment are 0.038%, while CO2 levels inside the human body, in particular in the lower respiratory tract, can reach 5% or more. The importance of this gaseous compound for *S. pneumoniae* is illustrated by the observation that the depletion of CO2 from ambient air completely inhibits pneumococcal growth (21). Moreover, about 8% of all clinical isolates require a CO2-enriched environment for growth in laboratory conditions (3). This intrinsic CO2 dependence of *S. pneumoniae* and many other (micro)organisms is most likely related to an anabolic need for CO2 or bicarbonate (HCO3⁻) during biosynthesis of nucleic acids, amino acids, and UFAs (1). Pathogens can often sequester CO2 directly from host tissues, but in the absence of sufficient levels of extracellular CO2, endogenous CO2 needs to be enzymatically fixed. Carbonic anhydrases

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(CAs; EC 4.2.1.1) are enzymes that catalyze the reversible reaction CO₂ + H₂O ⇌ HCO₃⁻ + H⁺. Because HCO₃⁻ cannot passively diffuse across biological membranes, its formation significantly delays the release of intracellular CO₂. At least five different classes of CAs have been described, and most eukaryotic, prokaryotic, and archaeal species express at least one CA class (39, 40).

Genome analysis (39) has revealed that S. pneumoniae has one putative CA, a β-class CA that is highly conserved in all available pneumococcal genome sequences. Pneumococcal CA (PCA) is highly homologous to CAs in other streptococcal species, such as Streptococcus pyogenes. The closest nonstreptococcal CA homologs are found in Mycobacterium species, while PCA homologs in other respiratory tract pathogens such as Neisseria meningitidis and Haemophilus influenzae are more divergent (40). The aim of this study was to investigate the functional characteristics of the pca gene and the encoded PCA enzyme in S. pneumoniae and to establish the relevance of PCA for pneumococcal growth and survival under CO₂-poor conditions in vitro. Further, we examined the importance of PCA during host-pathogen interaction.

### Materials and Methods

#### Bacterial strains and growth conditions.

The bacterial strains that were used in this study are listed in Table 1. S. pneumoniae strains were routinely grown under static conditions in GM17 broth (23) or on blood agar (BA) plates composed of Colombia agar (Oxoid) supplemented with 5% sheep blood (BioWhittaker). Cultures were incubated in a 5%-CO₂ incubator at 37°C. To compare growth under CO₂-poor and -rich conditions, mid-log-phase cultures of pneumococcal strains in CO₂-enriched GM17 were diluted 50-fold in medium that was exposed overnight to ambient air (0.038% CO₂) or to ambient air enriched with 5% CO₂, respectively. Pneumococcal genetic transformation was performed as described previously (10), and importantly, for preparation of competent S. pneumoniae strains lacking the pca gene (Δpca), all media were first exposed to ambient air enriched with 5% CO₂. For transformation of the CO₂-dependent carriage strains, a 1:1 mixture of competence-stimulating peptide 1 (CSP-1) (100 ng/ml) and CSP-2 (100 ng/ml) was used. Viable-bacteria counts were derived from CFUs after plating 10-fold serial dilutions in PBS. Escherichia coli strains were routinely grown at 37°C on Luria Bertani (LB) agar plates or in LB broth in a shaking incubator at 200 rpm. E. coli transformation was performed by the CaCl₂ competence method (35). Lactococcus lactis strains were routinely grown on GM17 agar plates or in GM17 broth as static cultures at 30°C. L. lactis transformation was performed by electroporation (23). The antibiotics and stock solutions used for complementation studies were ampicillin, 100 μg/ml; spectinomycin, 150 μg/ml; kanamycin, 500 μg/ml for S. pneumoniae and 50 μg/ml for E. coli; trimethoprim, 0.25 μg/ml; chloramphenicol, 2.5 μg/ml for S. pneumoniae and 5 μg/ml for L. lactis; and tetracycline, 5 μg/ml for L. lactis culture. For construction of directed-deletion mutants and glutathione S-transferase (GST) fusion protein cloning, the proofreading Pwo DNA polymerase (Roche) was used. For other PCR-based approaches, AmpliTaq DNA polymerase (Applied Biosystems) was applied. The primers (Biogene, Nijmegen, Netherlands) that were used in this study are listed in Table S1 in the supplemental material.

### Construction of pneumococcal mutants.

Directed-deletion mutants of S. pneumoniae were generated by allelic exchange of the target gene with an antibiotic resistance marker as described previously (10). Briefly, overlap extension PCR was applied to insert the kanamycin or spectinomycin resistance cassette of the wild-type strain, using chromosomal DNA of the mutant strains as the donor. The flanking sequences of all the target genes used in this study were PCR amplified with the PBNISPCA_L/H primer set.

### DNA extraction and PCR conditions.

Chromosomal DNA was isolated from S. pneumoniae and E. coli broth cultures by cetyltrimethylammonium bromide (CTAB) extraction as described previously (47). Plasmids were isolated from E. coli and L. lactis broth cultures with a Qiaprep mini- or midikit (Qiagen). For construction of directed-deletion mutants and glutathione S-transferase (GST) fusion protein cloning, the proofreading Pwo DNA polymerase (Roche) was used. For other PCR-based approaches, AmpliTaq DNA polymerase (Applied Biosystems) was applied. The primers (Biogene, Nijmegen, Netherlands) that were used in this study are listed in Table S1 in the supplemental material.

### Plasmid construction.

All plasmids used in this study are listed in Table 2. To obtain the plasmids for complementation of the CO₂-dependent growth defect of the Δpca strains, the pca gene of S. pneumoniae TIGR4 and the ecca gene (ECDH1B_106) of E. coli DH5a were PCR amplified with the PBNISPCA_L/H primer set.
TABLE 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristicsa</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pR410</td>
<td>Donor for kanamycin resistance cassette</td>
<td>43</td>
</tr>
<tr>
<td>pR412</td>
<td>Donor for spectinomycin resistance cassette</td>
<td>28</td>
</tr>
<tr>
<td>pCR2.1</td>
<td>Cloning vector; Ap’ Km’</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pGE1X-1N</td>
<td>Expression vector with N-terminal GST tag; Ap’</td>
<td>Novagen</td>
</tr>
<tr>
<td>pNG8048E</td>
<td>Expression vector with nisin-inducible promoter; Ca’</td>
<td>23</td>
</tr>
<tr>
<td>pWA1</td>
<td>pCR2.1 with pca gene, BamiHI site; Ap’ Km’</td>
<td>This study</td>
</tr>
<tr>
<td>pWA4</td>
<td>pGE1X-1N with gist-pca construct; Ap’ pCR2.1 with pca gene, BsaI site; Ap’</td>
<td>This study</td>
</tr>
<tr>
<td>pCR2.1-PCA_L</td>
<td>pCR2.1 with pca gene, BsaI site; Ap’ Km’</td>
<td>This study</td>
</tr>
<tr>
<td>pCR2.1-ECCA</td>
<td>pCR2.1 with ecca gene, BsaI site; Ap’ Km’</td>
<td>This study</td>
</tr>
<tr>
<td>pUO1</td>
<td>pNG8048 with ecca gene behind nisin-inducible promoter; Ca’</td>
<td>This study</td>
</tr>
<tr>
<td>pUO3</td>
<td>pNG8048 with pca gene behind nisin-inducible promoter; Ca’</td>
<td>This study</td>
</tr>
</tbody>
</table>

a Ap’, ampicillin resistant; Km’, kanamycin resistant; Ca’, chloramphenicol resistant.

PBNSIPCA_R and PB_NISECCA_R/LPB NISECCA_R primer pairs, respectively. The PCR products were cloned into the pCR2.1 cloning vector of a TA cloning kit (Invitrogen) to obtain pCR2.1-PCA_L and pCR2.1-ECCA, respectively. In the next step, the genes were excised by BsaI/EcoRI digestion and ligated to the NcoI/EcoRI-digested pG0848 plasmid to obtain pUO3 and pUO1, respectively. To obtain the plasmid for overproduction of GST-PCA, the pca gene of S. pneumoniae TIGR4 was PCR amplified with the PBPC/S_PBPBA_E primer pair and cloned into pCR2.1 to obtain pWA1. In the next step, the pca gene was excised by BamiHI/EcoRI digestion and subcloned behind the GST gene in a BamHI/EcoRI-digested pGE1X-1N vector to obtain pWA4. Cloning of the pCR2.1 and pGE1X-1N plasmids was performed in E. coli DH5α, and cloning of the pG0848E plasmids was performed in L. lactis NZ9000. The nucleotide sequences of the PCR products in the pCR2.1 plasmid were confirmed by sequencing.

Production and purification of recombinant GST-PCA. For GST-PCA production, an overnight culture of E. coli BL21 (pWA4) was diluted 50-fold in prewarmed (37°C) 2% LB supplemented with 0.5% glucose. At an optical density at 600 nm (OD600) of 0.6 to 0.8, 0.1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) was added, and cultures were shifted to room temperature. After 4 h, cells were plated on ice, pelleted by centrifugation, resuspended in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5], 300 mM NaCl, 0.5 mM dithiothreitol [DTT], 1.5 mM MgCl2, 0.2 mM EDTA, 1% Triton X-100) with 1× protease inhibitor mixture (Complete Mini; Roche Applied Science) to a cell density equivalent to an OD600 of 1.00, and lysed by sonication. Insoluble debris in the lysate was removed by centrifugation at 16,000 × g for 10 min at 4°C, and the supernatant was incubated overnight with prewashed (1× PBS) glutathione Sepharose 4 Fast Flow beads (GE Healthcare) at 4°C. Nonspecifically bound proteins were removed by washing the beads three times with lysis buffer for 15 min at 4°C. GST-PCA was eluted with elution buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 10 mM glutathione, and 0.5 mM DTT). The eluate was dialyzed against 50 mM Tris, pH 7.5. The protein concentration in the GST-PCA solution was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce).

Carbonic anhydrase activity assay. The activity of CAs was determined by the changing-pH/dye indicator method (22) on an RX.2000 rapid-mixing stopped-flow unit (Applied Photophysics, United Kingdom). Briefly, enzyme samples were diluted in reaction buffer at pH 7.5 (50 mM HEPES [pH 7.5], 200 mM phenol red, and 200 mM Na2SO4) or at pH 8.4 (50 mM TAPS [pH 8.4], 200 mM m-cresol purple, and 200 mM Na2SO4), and the reaction was initiated by the addition of an equivalent amount of CO2-saturated water. The subsequent restoration of CO2/HCO3− balance was monitored by the color conversion of the pH-sensitive dye indicators at 558 nm (pH 7.5) or 578 nm (pH 8.4). All reactions were performed at 25°C. The CA activities of GST-PCA and human CA II (HcALII Sigma) were measured at final concentrations of 100 μg/ml and 0.5 μg/ml, respectively. When appropriate, 50 mM Tris-HCl (pH 7.5) and dimethyl sulfoxide (DMSO) were included as nonsenzymatic controls. The stock solutions for CA inhibition studies were 100 mM acetazolamide ([AZA] Sigma) and 100 mM ethoxzolamide ([EZA] Sigma) in DMSO.

Cell lines, culture conditions, and host-pathogen studies. The human pharyngeal epithelial cell line Detroit 562 (CCL-138; ATCC) was routinely grown in RPMI 1640 medium without phenol red (Invitrogen, Netherlands) supplemented with 1 mM sodium pyruvate and 10% fetal calf serum (FCS). The human brain microvascular endothelial cell line (HBMEC) was cultivated in RPMI 1640 medium supplemented with 10% FCS, 10% Nu-Serum, 2 mM l-glutamine, 1 mM sodium pyruvate, 1% minimal essential medium (MEM)-vitamins, and 1% nonessential amino acids (42). Prior to infection, HBMEC monolayers were incubated for 1 h in culture medium with 10 ng/ml of tumor necrosis factor alpha (TNF-α). The murine macrophage-like cell line J774 (TIB 67; ATCC) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) (Invitrogen, Netherlands) with 10% FCS. All cells were cultured in a 5% CO2 incubator at 37°C.

Pneumococcal adherence, invasion, and intracellular survival studies were performed essentially as described previously (7, 11, 16). Briefly, monolayers of J774, Detroit 562, or HBMEC were infected with bacteria in 5% CO2-enriched culture medium with only 1% FCS (infection medium). Subsequently, the pneumococci were allowed to adhere to the cells for 0.5, 1, 2, or 4 h, respectively, and nonadherent bacteria were removed by washing. To quantify adherence, host cells were detached from the wells and lysed with 0.025% Triton X-100 or 1% saponin and trypsin-EDTA (0.05%-0.02%). To determine the level of invasion into the host cells, extracellular S. pneumoniae cells were killed by a 1-h incubation with 1 mM 5% CO2-enriched infection medium supplemented with gentamicin (10 μg/ml) and penicillin G (10 μg/ml) before cell lysis. To examine intracellular survival, cells were infected and treated with gentamicin and penicillin G as described above, after which cells were washed once and fresh 5% CO2-enriched medium containing gentamicin (13.34 μg/ml) and penicillin G (0.67 μg/ml) (1/15 of beginning antibiotic concentration) was added to each well for prolonged incubation. For all in vitro cell culture studies, the pneumococcal wild-type and mutant strains grew comparably in infection medium alone. Results were corrected mathematically to account for small differences in count in the initial inoculum.

In vitro colonization and bacteremia experiments. Bacteremia and nasopharyngeal colonization experiments with mice were conducted with 9-week-old female outbred CD-1 mice (Harlan, Horst, Netherlands) as described recently (18). Briefly, for the colonization experiments, 1 × 106 CFU in 10 μl of PBS was administered to the nostrils of groups of five mice for each strain, and bacteria were recovered from the nasopharynx by flushing the nose with 2 ml of sterile PBS at 96 h. Bacteremia experiments were performed twice with groups of at least five mice for each strain. Mice were infected intravenously in the tail vein with 1 × 105 CFU in 100 μl of PBS, and bacteria were recovered from the blood by retro-orbital puncture. For mice in which no bacteria were found, a lower limit of detection (22 CFU/ml) was used. Results were corrected mathematically to account for small differences in bacterial count in the initial inoculum. All experiments were performed with the approval of the Animal Experimentation Committee (DEC) of the Radboud University Nijmegen Medical Centre.

In silico analysis. The subcellular location of PCA enzyme was predicted by various online prediction servers, such as PSORTb (http://www.psort.org) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM). Conservation of the pca gene and PCA protein was performed by the genomic BLAST service on the website of the National Center for Biotechnology Information (NCBI) of the U.S. National Library of Medicine (http://www.ncbi.nlm.nih.gov/).

Statistical analysis. For in vitro host-pathogen studies, data were analyzed using an unpaired Student’s t test, with P values of <0.05 considered significant. All statistical analyses were performed using GraphPad Prism version 4.0.

RESULTS

The pca gene is required for pneumococcal growth under CO2-poor conditions. To determine the importance of the pca gene for pneumococcal growth, pca deletion mutants (∆pca) were constructed from three S. pneumoniae strains, i.e., R6 (∆spr0026), D39 (∆SPD_0030), and TIGR4 (∆SP_0024). All ∆pca strains were able to grow normally on BA plates and Trypticase soy broth (TSB) agar plates supplemented with catalas (Trypticase soy agar [TSA]) under ambient air enriched with 5% CO2 (data not shown). In vitro growth rates in
5%-CO2-enriched GM17 broth medium were similar for the \( H_9004 \) pca and wild-type strains, with cultures reaching an OD$_{620}$ of 0.3 or more (Fig. 1A, left panel). In GM17 broth medium that was exposed to ambient air, the wild-type strains were also able to reach a high OD$_{620}$. In contrast, growth of all \( H_9004 \) pca strains under these CO$_2$-poor (0.038%) growth conditions was attenuated, and cultures did not reach an OD$_{620}$ above 0.1 (Fig. 1A, right panel). Growth of the \( H_9004 \) pca strains under CO$_2$-poor conditions was also impaired on TSA plates and reduced on BA plates (data not shown).

To exclude polar effects due to disruption of the pca gene, we provided the pca gene in trans on the pUO3 plasmid behind a nisin-inducible promoter. Induction of pca gene expression by the addition of nisin restored growth of the nisin-responsive \( R_6 \) bga::nisRK pca (pUO3) strain in CO$_2$-poor GM17 broth (Fig. 1B). Introduction of the pUO1 plasmid with the gene for \( E. coli \) carbonic anhydrase (ECCA) (12) into \( R_6 \) bga::nisRK pca also reversed the CO$_2$ dependence of this strain (Fig. 1B). Interestingly, complementation by ECCA did not appear to require induction with nisin. Because pUO1 could not restore the CO$_2$ dependence of the \( R_6 \) pca strain lacking the NisRK sensor for nisin (data not shown), it is likely that autoinduction of the NisRK two-component signal transduction system resulted in expression of small but sufficient amounts of ECCA.

**PCA has carbonic anhydrase activity.** The PCA enzyme was further characterized with enzymatic activity and inhibition assays. To facilitate the measurement of PCA enzymatic activity, PCA was overproduced as a GST fusion protein in \( E. coli \). Since no endogenous \( E. coli \) CA activity was detected in the lysates of control cells expressing only the GST protein, the CA activity in \( E. coli \) cells expressing GST-PCA can be fully ascribed to the presence of the recombinant protein (data not shown). The affinity-purified recombinant GST-PCA protein catalyzed the conversion of CO$_2$ to HCO$_3^-$ at pH 8.4, whereas the enzymatic activity was almost completely abrogated at pH 7.5 (Fig. 2A). Sulfonamides such as AZA and EZA are broad-range CA inhibitors that are active against most CAs (39), including the homologous Rv1284 CA in *Mycobacterium tuberculosis* (29). Interestingly, the presence of 100 \( \mu \)M AZA or 100 \( \mu \)M EZA did not reduce the CA activity of recombinant GST-PCA, whereas that of hCAII was completely inhibited (Fig. 2B).
2B). Since both compounds also did not induce CO₂ dependence in \textit{S. pneumoniae} wild-type strains (data not shown), these sulfonamides are unlikely to have high affinity for PCA.

**PCA is linked to UFA biosynthesis.** The biosynthesis pathways for nucleic acids, fatty acids, and several amino acids all contain an essential HCO₃⁻-dependent carboxylation step that could potentially account for the observed growth defect of microbial CA mutants in CO₂-poor conditions (1). To investigate if one or more of these carboxylation steps are responsible for the growth inhibition of the \textit{S. pneumoniae} Δ\textit{pca} strains in CO₂-poor GM17 broth medium, we complemented pneumococcal cultures with sodium hydrogen carbonate (NaHCO₃) or various metabolic intermediates (i.e., adenine, uracil, arginine, aspartic acid, palmitic acid [in 0.1% Tween 40], or oleic acid [in 0.1% Tween 40]) (Fig. 3A). As predicted, NaHCO₃ fully reversed growth of the \textit{S. pneumoniae} TIGR4Δ\textit{pca} strain under CO₂-poor conditions. The unsaturated fatty acid (UFA) oleic acid was the only metabolic intermediate that could partially restore growth as well, although not to the same level as NaHCO₃. Other sources of UFAs, such as Tween 20 and Tween 80 (30), could also (partially) reverse the CO₂ dependence of the \textit{S. pneumoniae} TIGR4Δ\textit{pca} strain (Fig. 3B). In contrast, the saturated fatty acid (SFA) palmitic acid (Fig. 3A) or Tween 40 (Fig. 3B), which is a Tween derivative that is solely composed of SFAs, was ineffective.

Because supplementation with SFAs could not reverse the CO₂-dependent growth inhibition of the Δ\textit{pca} strains, CO₂ fixation by PCA appears to be essential when insufficient UFAs are available in the growth medium. The synthesis of UFAs and SFAs in \textit{S. pneumoniae} occurs essentially by the same pathway (27). The dependency of the Δ\textit{pca} strains on UFA supplementation for growth under CO₂-poor conditions therefore suggests that under this condition UFAs are more readily depleted. Recently, it was reported that the reactive oxygen species (ROS) scavenger salicylate increased the unsaturation index of bacterial-membrane fatty-acyl chains under aerobic (thus CO₂-poor) growth conditions by protecting UFAs against endogenous oxidative stress (31). In line with this observation, cultures of the \textit{S. pneumoniae} R6Δ\textit{pca} and TIGR4Δ\textit{pca} strains grown under CO₂-poor conditions reached an almost-2-fold-higher optical density when supplemented with salicylate (Fig. 4A). Neutralization of endogenous H₂O₂, which also plays an important role in lipid peroxidation (38), through the addition of high concentrations of catalase restored growth of the \textit{S. pneumoniae} R6Δ\textit{pca} and TIGR4Δ\textit{pca} strains to an almost-3-fold-higher optical density (Fig. 4B). Despite the involvement of pyruvate oxidase (\textit{spxB}) in endogenous H₂O₂ production (41), disruption of the \textit{spxB} gene in the TIGR4Δ\textit{pca} and R6Δ\textit{pca} strains did not restore growth to the same level as that in the catalase-complemented cultures (Fig. 4B). Moreover, the addition of catalase still promoted growth of the \textit{S. pneumoniae} TIGR4Δ\textit{spxBΔpca} and R6Δ\textit{spxBΔpca} strains (Fig. 4B).

**PCA is required for intracellular survival inside host cells.** Membrane fatty acids are essential for pneumococcal growth and survival (26) and are important targets for host defense mechanisms (38). Because our experiments suggest that PCA activity and UFA biosynthesis are linked, we investigated the specific contribution of PCA to pneumococcal host-pathogen interactions. To identify PCA-mediated effects on the interaction of \textit{S. pneumoniae} with host cells, we assessed the ability of the Δ\textit{pca} strains to adhere to, invade, and survive in different cell lines. First, we studied the interaction of \textit{S. pneumoniae} with human pharyngeal epithelial Detroit 562 cells, which are representative of the host cells encountered by \textit{S. pneumoniae} during colonization of human upper airways. Disruption of the \textit{pca} gene in the unencapsulated (Δ\textit{cps}) derivative of the \textit{S. pneumoniae} TIGR4 strain did not lead to decreased adherence to (Fig. 5A) or invasion of (Fig. 5C) these epithelial cells. However, at 1 h after pneumococcal invasion of the host cells, we observed a statistically significant 1.3-fold reduction in intracellular survival of the TIGR4Δ\textit{cpsΔpca} strain in Detroit 562 cells (Fig. 5E). Next, we examined the role of PCA during interaction of \textit{S. pneumoniae} with the HBMEC line. Endothelial cells are the main component of the blood-brain barrier, and penetration of this barrier by pathogens can lead to men-
ingritis. Adherence to HBMEC was not significantly different between the TIGR4Δcps and TIGR4ΔcpsΔpca strains (Fig. 5A). In contrast, the number of viable intracellular bacteria that could be recovered from HBMECs directly after uptake by the macrophages was 2-fold lower for the D39ΔcpsΔpca strain than for the D39Δcps strain (Fig. 5D). Moreover, temporal monitoring revealed that phagocytic killing of intracellular bacteria continued to be significantly faster for the D39ΔcpsΔpca strain than for the D39Δcps strain (Fig. 5G). Despite the in vitro contribution of PCA to pneumococcal intracellular survival, no significant difference between the S. pneumoniae TIGR4 wild-type and TIGR4Δpca strains was observed in mouse models of pneumococcal nasopharyngeal carriage and bacteremia (see Fig. S1 in the supplemental material).

The pca gene is present in CO₂-dependent circulating strains. The pca gene appears to be a highly conserved gene, which is present in all 11 complete and 18 draft S. pneumoniae genomes that are currently available in the public databases. Still, about 8% of all S. pneumoniae isolates from various sources have been reported as CO₂ dependent (3). To exclude the possibility that the pca gene is absent in these circulating strains, we investigated whether the CO₂ dependence of these isolates is related to the absence of a functional pca gene. Two out of 126 carriage strains (H23 and H26) isolated from healthy Venezuelan children (our unpublished data) did not grow on BA and TSA plates unless the environment was enriched with 5% CO₂. PCR analysis indicated that the pca gene was present in both CO₂-dependent strains (Table 3), and genetic transformation of these strains with chromosomal DNA from both the S. pneumoniae R6 wild-type and R6Δpca strains resulted in CO₂-independent colonies (Table 3). These results suggest that the observed CO₂ dependence of the H23 and H26 strains is associated with a genetic defect or a missing gene other than pca. In addition, further phenotypical characterization of these strains showed that their CO₂ dependence was different from that of the S. pneumoniae Δpca strains used in this study. Although both strains were completely CO₂ dependent for growth on BA plates, the H23 strains reached high optical densities in CO₂-poor GM17 broth medium. In contrast, the H26 strain did not grow at all in CO₂-poor GM17 broth medium, not even when it was supplemented with UFA (Tween 80) (Table 3).

**DISCUSSION**

The respiratory tract pathogen S. pneumoniae needs to adapt to the various conditions it encounters during transmission, colonization, and disease. Currently, relatively little is known about the genetic and metabolic factors that contribute to an adequate response of this bacterium to changes in CO₂ availability. In this study, we showed that the putative carbonic anhydrase in S. pneumoniae has an important role for growth in CO₂-poor conditions.

Our experiments clearly showed that the pca gene encodes a functionally active carbonic anhydrase. All the Δpca strains were growth deficient in CO₂-poor conditions but could be complemented by the addition of HCO₃⁻, the expected end product of PCA enzymatic activity. In addition, growth of the Δpca strains could be restored by in trans expression of the
well-characterized homologous β-CA (ECCA) from *E. coli*. Finally, recombinant GST-PCA was able to catalyze the conversion of CO$_2$ to HCO$_3^\text{-}$ (13). Interestingly, PCA did not appear to be active at the physiological pH of 7.5. This is not unusual for β-CAs and has been observed for ECCA and the *H. influenzae* CA (HICA). Most likely, this pH-dependent behavior is linked to the pH-dependent coordination of Zn$^{2+}$ in the active site (13). Furthermore, both ECCA and HICA appear to have an alternative bicarbonate binding site that renders the enzyme inactive at physiological pH when sufficient substrate is present (13). Although PCA appears to miss essential amino acids that form the alternative bicarbonate binding site, it is also not unlikely that differences exist between its CA activity in enzymatic assays and in physiological conditions. Another striking characteristic of PCA is its lack of affinity for broad-range carbonic anhydrase inhibitors. This indicates that this enzyme is deviant from other well-characterized CAs, which is not surprising, as there is huge variation among the different CAs, and CA inhibitors were often developed against unrelated hCAs. In fact, differences between PCA and hCAs could benefit the therapeutic potential of PCA inhibitors.

<table>
<thead>
<tr>
<th>Strain$^a$</th>
<th>Transformation (CFU/ml) with DNA from:</th>
<th>Growth on or in indicated plate or broth$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R6</td>
<td>R6Δ* pca</td>
</tr>
<tr>
<td>H23</td>
<td>19,800</td>
<td>− 14,900</td>
</tr>
<tr>
<td>H26</td>
<td>67</td>
<td>67</td>
</tr>
</tbody>
</table>

$^a$ Both strains carry the *pca* gene.

$^b$ No. of colonies growing on BA plates under CO$_2$-poor conditions.

$^c$ +, growth; −, no growth.

To the metabolic complementation experiments revealed, in analogy to the role of CAs in other microorganisms (1, 5), that the cellular function of PCA in CO$_2$-poor conditions is at least linked to FA biosynthesis. This implies that PCA provides HCO$_3^\text{-}$ re-
quired for the carboxylation of acetyl coenzyme A (acetyl-CoA) by acetyl-CoA carboxylase to form malonyl-CoA, which is the first committed step of FA biosynthesis (15). We did not observe a stimulating effect of any of the other tested metabolic intermediates on the growth of the S. pneumoniae Δpca strains in CO2-poor GM17 broth medium. This implies that GM17 medium contains limiting amounts of UFAs but sufficient levels of the other metabolites to support growth. Based on the UFA supplementation experiments and previous observations of other microorganisms (1, 5), we can predict that other carboxylation reactions, e.g., those involved in biosynthesis of some amino acids, pyrimidines, and purines, also depend on PCA activity when CO2 levels are low. Still, we feel that support of UFA biosynthesis is one of the most relevant aspects of PCA function. Although S. pneumoniae is able to tolerate low levels of membrane SFAs, insufficient UFAs lead to decreased cell viability (2). In ambient-air conditions, both environmental and cellular UFAs are prone to oxidation and can be replaced only by the PCA-supported de novo biosynthesis of UFAs. In addition, endogenous production of ROS by S. pneumoniae itself leads to increased cellular UFA peroxidation (31). Due to the transient phenotype of the pca mutation, it was not possible to perform a straightforward experiment to directly link the disruption of the pca gene to an alteration in the membrane FA composition or increased ROS sensitivity. In the absence of CO2, the Δpca strains do not grow, whereas in the presence of CO2, the Δpca and wild-type strains are phenotypically identical. In analogy with studies of S. pneumoniae UFA auxotrophs (27), we did attempt to complement cultures of the Δpca strains in CO2-poor conditions with UFAs to restore growth and allow characterization of membrane FAs. However, supplementation of cultures of the pneumococcal wild-type and Δpca strains with UFAs completely repressed expression of the FA biosynthesis gene cluster (our unpublished data), which inevitably results in a membrane that is predominantly composed of exogenous FAs (9).

It is tempting to speculate about the role of PCA in neutralizing the detrimental effect of pneumococcal SpxB activity. In ambient air, SpxB produces H2O2, acetyl-phosphate, and CO2. Production of H2O2 leads to UFA peroxidation (31), whereas acetyl-phosphate can readily be converted to acetyl-CoA by phosphate acetyltransferase to support de novo FA biosynthesis. PCA then acts to convert CO2 to HCO3−, allowing carboxylation of acetyl-CoA to form malonyl-CoA. Currently, this hypothesis is not supported by our own observations, as catalase improved growth of both the S. pneumoniae Δpca and ΔpcaΔspxB strain cultures. However, the interconnection between SpxB activity and FA biosynthesis is still poorly understood and might involve different metabolic and regulatory pathways (31, 44). Alternatively, this suggests that other sources of endogenous oxidative stress, such as the Fenton reaction (31) or lactate oxidase activity (44), have a profound impact on the growth arrest of the Δpca strains in CO2-poor conditions as well.

The role of PCA in the de novo biosynthesis of UFAs and, possibly, other metabolites could also explain the decreased viability of the S. pneumoniae Δpca strains after invasion of endothelial cells and uptake by macrophages. During phagocytosis, and possibly endocytosis (33), a substantial portion of the intracellular bacteria is sorted to the host-cell lysosome. The low pH of this compartment reduces HCO3− availability, and the production of ROS leads to peroxidation of bacterial membrane UFAs (38) and nucleic acids (37). Interestingly, the effect of pca disruption on S. pneumoniae invasion and intracellular survival inside Detroit 562 pharyngeal epithelial cells was not as pronounced as in the two other cell types. Whether this difference reflects on the different routes for pneumococcal invasion of Detroit 562 cells by interaction with the polymeric immunoglobulin receptor (pIgR) (49) and HBMECs by interaction with the platelet-activating factor receptor (PAFr) (34) remains to be studied. A role for microbial carbonic anhydrases inside host cells was earlier suggested for a Salmonella enterica serovar Typhimurium CA (mig-5), which was expressed after uptake in macrophages and a mutant of which had a marked decrease in spleen colonization of mice (46). In contrast to findings for Salmonella CA mutants, we were not able to link PCA with virulence in animal models of bacteremia. However, this observation is in line with the outcome of a previous study showing that mice deficient in the NADPH oxidase subunit gp91, which is essential for lysosomal ROS production, were as sensitive to pneumococcal infection as wild-type mice (36). Furthermore, it is known that pneumococcal capsular polysaccharides prevent recognition and uptake of the bacterium by host immune cells, and once S. pneumoniae remains extracellular during infection of the blood, it might utilize serum HCO3− and FAs (9). Possibly, the role of PCA in pneumococcal disease is more pronounced in animal models of disease in which the bacterium needs to traverse the boundaries of epithelial and endothelial cells for dissemination from the respiratory tract to the blood and cerebrospinal fluid.

Finally, the role of PCA in S. pneumoniae can be projected onto CAs in other (respiratory tract) pathogens. Although CAs are ubiquitous enzymes in many microorganisms, most studies have investigated the role of CAs that are exposed to the surface or periplasm, have species-specific functions, or do not belong to the class of β-CAs (39). Here, we show that cytosolic β-CAs related to PCA are involved in FA biosynthesis and may offer novel opportunities for the design of broad-range therapies. Furthermore, PCA is probably only one of the factors that contribute to the adaptation of S. pneumoniae to CO2-poor conditions, which might be relevant for pneumococcal transmission in environmental ambient air. Detailed examination of the metabolic pathways that depend on PCA-mediated CO2 fixation and the identification of the genetic basis for the CO2 dependence observed in approximately 8% of all circulating pneumococcal isolates is expected to lead to novel insights into the way respiratory pathogens adapt to the CO2- and HCO3−-poor environments they encounter during transmission, colonization, and disease.

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